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Specification and Drawings, as originally filed, with Application for Patent Serial No: **2,306,315**, on April 20, 2000, by **IMI INTERNATIONAL MEDICAL INNOVATIONS INC.,** for "Non-Invasive Cholesterol Test".

Agent certificateur/Certifying Officer

February 5, 2003

Date

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ABSTRACT OF THE DISCLOSURE

A skin cholesterol test is provided, in which liquid or semi-solid reagents are applied to a patient's skin, to bind to the skin cholesterol, followed by development of color in the reagents, the degree of color development being directly related to the quantity of cholesterol in the skin. Instead of visual assessment, however, the liquid or semi solid reagents in which the color has been developed are analyzed colorimetrically, to determine degree of color development from which cholesterol levels can be at least semi-quantitatively obtained. The chosen colorimetric parameters, such as hue angle or shade, are independent of color density, intensity or lightness, and simply measure the color shade. This essentially eliminates the uncertainties introduced from the background color of the skin, so that the test can be conducted on the patient's skin surface. Instrumental colorimetric (spectrophotometric) analysis produces objective numbers which are at least semi-quantitative and indicative of cholesterol levels of the patient.

NON-INVASIVE CHOLESTEROL TEST

FIELD OF THE INVENTION

This invention relates to diagnostic methods applicable to human patients, and more particularly to tests for estimating a patient's skin cholesterol level.

BACKGROUND OF THE INVENTION

The association of high serum cholesterol levels in patients with propensity to develop atherosclerosis and consequent increased incidence of coronary heart attack, stroke and PVD is firmly established, so that frequent monitoring of patient's cholesterol levels is desirable. More commonly, cholesterol level is determined from extracted blood samples. Many other diagnostic tests are commonly performed also on extracted blood samples, but most of these need only be conducted at longer intervals than cholesterol tests. The invasive nature of the blood collection procedure for cholesterol analysis discourages many patients from undergoing cholesterol monitoring as frequently as is advisable. Accordingly, there is a need for a non-invasive cholesterol test.

It is estimated that the skin contains about 11% of the body's total cholesterol, resulting largely from epidermal steroidogenesis and cholesterol diffusion from blood vessels. It has been postulated that the level of skin cholesterol may more accurately reflect the extent of atherosclerosis than the amount of serum cholesterol.

REFERENCE TO THE PRIOR ART

Nikitin, YP, Gordenko, I.A., Dolgov, A.V. and Filimonova, T.A., "Cholesterol Content in the Skin and its Correlation with Lipid Quotient in the Serum in Normals and in Patients with Ischemic Cardiac Disease", Cardiology 1987 II, No. 10, page 48-51, and others, have demonstrated that there is a close

correlation between cholesterol content in the arterial wall and cholesterol content in the skin of a patient. This suggests a possibility of developing skin tests to determine a patient's cholesterol level. The method described by Nikitin et al., however, involves removing and analyzing skin samples in vitro, a method which is impractical in a clinical setting.

U.S. Patents 5,489,510 and 5,587,295 Lopukhin et al., describe a non-invasive diagnostic test which is performed on the surface of the patient's skin, and which indicates skin cholesterol levels. In the test described in these patents, reagents are provided in the form of affino-enzymatic compounds which are bi-functional in their nature. The bifunctional compound A-B includes a binding agent A which is capable of discriminately forming stable complexes with cholesterol of the skin in order to give the whole bi-functional compound an affinity to cholesterol (for example digitonin); and a visualizing agent B, for example an enzyme such as peroxidase, which permits detection of the bi-functional compounds bound to skin cholesterol. In the practice of this test, a complex of a binding agent A and a visualizing agent B, optionally in combination with a bridging agent C to enhance the sensitivity of the test, i.e. a bi-functional conjugate A-C-B, may be placed on the skin of the palm of the patient. Bridging agent C is suitably a high molecular weight polyfunctional compound such as a polysaccharide or a protein, and serves to space the visualizing agent from the binding agent to minimize steric hindrance of the cholesterol-binding agent reaction. After a suitable incubation period to ensure binding of the complex to the cholesterol of the skin, the area is fully rinsed with clean water to remove unbound reagents. Then the binding area is treated with indicating agent D, to react with visualizing agent B so as to develop color. The greater the cholesterol level, the greater the degree of binding of the bi-functional compound to the skin, and the greater the degree of color development.

A cholesterol test based on the aforementioned patents of Lopukhin et al. has been developed commercially and put into commercial practice. It involves the provision of a kit comprising reagents and a color chart or r ader. Most of the reagents are contained in a vial, which the user applies to the test

area, on the palm of the hand, after removing the protective covers. After incubation, the user applies indicating agent and visually assesses the degree of color change which occurs, alongside the color chart or using the reader.

One disadvantage of such a test is the requirement for visual assessment of the color changes. Whilst it is convenient that the test can be carried out by un-skilled personnel, such as the patient, the visual assessment of the resultant color change is subjective and essentially non-quantitative. It can give a valuable indication of cholesterol levels and hence potential problems, but not the type of quantitative measurements which a prescribing physician commonly prefers. The assessment is easily influenced by the nature and color of the background, namely the skin.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide a novel diagnostic, non-invasive test for cholesterol.

It is a further and more specific object to provide such a test which is capable of producing at least semi-quantitative results.

The present invention provides a cholesterol test in which liquid or semi-solid reagents are applied to a patient's skin, to bind to the skin cholesterol, followed by development of color in the reagents, the degree of color development being directly related to the quantity of cholesterol in the skin. Instead of visual assessment, however, the liquid or semi solid reagents in which the color has been developed are analyzed colorimetrically, to determine degree of color development from which cholesterol levels can be at least semi-quantitatively obtained. The chosen colorimetric parameters, such as hue angle or shade, are independent of color density, intensity or lightness (L), and simply measure the color shad . This essentially eliminat s the uncertainties introduced from the background color of the skin, so that the test can be conducted on the patient's skin surface. Instrumental

colorimetric (spectrophotometric) analysis produces objective numbers which are at least semi-quantitative and indicative of cholesterol levels of the patient.

Thus according to the present invention, from one aspect, there is provided a process of determining skin cholesterol levels of a patient, which comprises:

applying to a person's skin surface a reagent which selectively binds to skin cholesterol;

causing color developing chemical reaction with the skin cholesterol-bound reagent combination so formed, to form a colored complex;

and subjecting the colored complex so formed to spectrophotometric analysis to read therefrom a pre-defined characteristic of the color of the colored complex.

A further aspect of the invention is a kit for determination of skin cholesterol levels of a human patient, and comprising:

a source of detecting agent, capable of binding to skin cholesterol of the patient to form a bound combination therewith on the skin;

a source of visualizing agent, capable of reacting with the detecting agent - binding agent bound combination to form an optically altered complex therewith;

a source of developing agent and means for applying the developing agent to the optically altered complex, to develop color therein;

and means for confining and for presenting said optically altered complex to a portable reflectance spectrophotometer to determine therefrom a defined color characteristic selected from hue angle, chroma or saturation.

BRIEF REFERENCE TO THE DRAWINGS

Figure 1 is a diagrammatic illustration of a test strip for use in the present invention;

Figure 2 is an illustration of a spectrophotometer reader in use in the present invention, and in its open position;

Figure 3 is a view similar to Fig. 2, but with the spectrophotometer in the closed position;

Figure 4 is a detail view of the lower portion or shoe of the spectrophotometer shown in Figure 2 and Figure 3.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The preferred process of the present invention employs liquid or semi-solid biochemical reagents, develops color therein determinative of the patient's skin cholesterol content, and subjects the color so developed to spectrophotometric analysis. According to the invention, the precise nature and identity or shade of the color so developed, as characterized for example by its hue angle, correlates with the amount of bound complex formed and hence with the cholesterol content of the skin. This measurement of color characteristic is objective and at least semi-quantitative. Accordingly, it is read independently of background skin color and is not influenced thereby, to any significant extent.

It is usually convenient to add all the reagents, in the appropriate order, to the patient's skin surface, allow color development on the skin surface, and then examine the color-developed complex spectrophotometrically, while it remains on the skin. The entire test can be conducted in under five minutes. The area of skin chosen for the test should be one which is essentially free from sebaceous glands, since such glands contribute cholesterol-containing sebum which would interfere with the results. The sole of the foot and the palm of the hand are suitable such skin areas, with the palm of the hand being the most convenient for use in the present test.

The kit includes a means for confining and presenting the color-developed complex for analysis by a portable spectrophotometer. Suitably this is a container in the form of a skin-adherent strip, with one or more wells passing

therethrough, so that reagents contained in the wells can contact the patient's skin. Container design will largely be dictated by the physical characteristics of the spectrophotometer. Instead of a container for the reagents, an inert thixotropic agent can be included with the reagents, to limit their spread across the skin surface and to prevent the mixing of test reagents with control reagents applied to adjacent skin locations.

Suitable spectrophotometers for use in the present invention are portable, reflectance-based, and give accurate measurements of color characteristics such as hue angles, lightness and chroma or saturation, when the incident light of the spectrophotometer is reflected back from the stained sample to the instrument's receiver. They are commercially available. A specific example of a suitable such instrument is that marketed by X-Rite, Grand Falls, Michigan, U.S.A as "Model CA22 Spectrophotometer. It is supplied with appropriate software so that it can be connected to a computer to give an accurate read-out of the hue angle of the stained sample under test. The spectrophotometer receives reflectances over the approximate wavelength 400-700 nm, i.e. over most of the visible light spectrum, suitably over about 20 nm intervals.

It is known that color may be defined and expressed in terms of hue angle. The concept of "hue angle" is defined and discussed in standard textbooks such as "Principles of Color Technology," by Fred W. Billmeyer and Max Saltzman, published by John Wiley and Sons (see particularly Chapters 1 and 2), incorporated herein by reference. "Hue" is the color or shade of a specimen independent of its brightness or intensity, and "hue angle" of a color or shade is the definition of its reflectance wavelength by angular position with reference to a standard three dimensional ellipsoidal continuum plot of the entire spectrum of visible light. The visible light (color) continuum is represented on an angular scale from 0 to 360°, and the angular values as read by the reflectance spectrophotometer are transformed into linearized form to give the transformed "hue angle" used in the process of th present invention. It is a featur of th present invention that hue angl of the colored complex bound to the skin

cholesterol correlates with skin cholesterol content.

Suitable chemical reagents for use in the present invention are generally those described in the aforementioned Lopukhin et al. patents, the disclosures of which are incorporated herein by reference. Their precise choice is not an essential or limiting feature of the present invention, provided that theie use in combination with one another results in the chemical development of color as a result of binding to skin cholesterol. The term "binding" is used herein in its broad sense of chemical reaction to cause attachment of one chemical entity to another, as well as specific affinity-type "gripping" interaction often encountered in biochemical systems.

Thus, the binding agent A is selected from a group of substances capable of discriminately forming stable complexes with free cholesterol of the skin in order to give the whole bi-functional compound in which it is involved an affinity to cholesterol. It can form a stable complex by direct reaction with cholesterol, before or after it is chemically attached to the visualizing agent B directly or through the bridging agent C.

Representative classes of compounds suitable as cholesterol binding agents A include:

steroid glycosides, containing as an aglycone a cyclopentaneperhydrophenanthrene fragment of the furostanole or spirostanole series, and an oligosaccharide fragment including 3 to 10 monosaccharide residues with linear or branched structures (Hinta P.H. "Structure and biological activity of steroid glycosides of spirostan and furostan series", Hishinev, Stinza, 1987, pg. 142) specific preferred examples of which are funcosides C, D, E, F, G and I, dioscin, rocosides C, D and E, lanotigonine, digitonin and tomatine;

triterpene glycosides, containing an aglycone of alpha or beta-amyryl, lupane, hopane, dommarane, linostane or holostan series, and

oligosaccharides comprising saccharide residues of branched or linear structure (Deknnosidze G.E., Chirva V.Y., Sergienko T.V., Uvarova N.L. "Study on Triterpene Glycosides", Tbilisi, Mesniereba, 1982;

hydrophobic proteins capable of discriminately forming a complex compound with cholesterol (Himov A.N., Titova G.V., Kozhevnikov H.A., Biochemistry, 1982, Vol. 47, No. 2, pg. 226-232); Himov A.N., Hozhevnikov H.A., Klyueva N.N. et al. Voprosy Meal., Hhimii, 1984, Vol. 30, No. 3, p. 86-90; Titova G.V., Hilyueva N.N., Hozhevnikov H.A., et al. Biochemistry, 1980, Vol. 45, No. 1, pg 51-55);

protein toxins, capable of discriminantely forming complex compounds with cholesterol. They are obtained from bacteria, marine microorganisms, insects of snakes (Dalin M.V., Fish N.G. "Protein Toxins or Microorganisms", Moscow, Medicine, 1980); or

polyens antibiotics, capable of discriminantely forming complex compounds with cholesterol (I.J. Katzenstein, A.M. Spielvogel, A.W. Norman, J. Antibiot., 27, 12, 1974, pg. 943-951; Jong Shan Shyng, Wang Hsi-Hua, Clin. J. Microbiol., 1976, 9, (1-2), pg. 19-30; Readio Josphine D. et al. Biochim. Biophys. Acta, 1982, 685 (2), pg 219-24); or

high affinity enzymes, whose substrate is cholesterol, and which have a high affinity to it. All of the above-mentioned publications are incorporated herein by reference.

The most preferred choice for cholesterol binding agent A is digitonin.

Visualizing agent B is commonly an enzyme, since enzyme/substrate reactions resulting in a color change are particularly us ful. Specific xamples of suitable such enzymes includ acetyl choline sterase, tyrosinas , glucose-6-

phosphate dehydrogenase, glucose oxidase, glucoamylase, beta-D-galactosidase, peroxidase, alkaline or acid phosphatase, alpha-chymotrypsin, and pyrophosphatase. Peroxidase is a preferred choice e.g. horseradish peroxidase (HRP).

The use of a bridging agent C enhances the technical performance of the method, and facilitates the production of the resulting, desirable A-C-B complex from which color can be developed, whilst preserving the functional activity of agents A and B. The most preferred A-C-B complexes are those which use steroid glycosides, which contain as an aglycone, a cyclopentaneperhydrophenanthrene fragment from the furostanol or spirostanol series and oligosaccharide fragments including two to 10 monosaccharides residues with linear or branched structures such as digitonin as the cholesterol affinity binding agent A. It is particularly desirable to use a bridging agent C when digitonin is chosen as the cholesterol binding agent A and HRP is chosen as the visualizing agent, since HRP is a relatively large molecule which, if bound directly to digitonin, might sterically hinder the reaction of digitonin with skin cholesterol. As bridging agent C for such purposes, it is preferred to use high molecular weight polyfunctional compounds. Their use allows a wide range of control over the proportion of agents A and C in the final complex. Such high molecular weight polyfunctional bridging agents C may be various polysaccharides, proteins or synthetic polymers, i.e. any suitable high molecular weight compound containing primary amine, carboxyl, hydroxyl, aldehyde, haloidanhydride, mixed anhydride, iminoester, azide, hydroxide, maleimide, isocyanate, or epoxide functional groups. Copolymers of acrylic acid or maleic acid or maleic anhydride and N-vinylpyrrolidone are the most preferred high molecular weight polyfunctional bridging agents C. Asymmetric low molecular weight bi-functional compounds such as bromocyan, trichlorotriazine or 2-amino-4,6-dichloro-3-triazine can also be used.

Indicating agent D typically contains a substrate of the enzyme mployed as the visualizing agent B, and additional compounds needed to make

the reaction between the enzyme and its substrate visible. A specific example of such an indicator agent D, for use with peroxidase enzyme as visualizing agent B, is an agent containing hydrogen peroxide, N,N-diethyl-p-phenylidene sulfate, together with appropriate stabilizers. Indicator agent D is selected in combination with visualizing agent B, from the range of compounds known in the art which will generate color developing reactions in combination with the chosen enzyme.

For conducting the test according to the invention, a kit is provided. The kit includes the required reagents in appropriately sealed packages such as vials or bottles equipped with droppers, a container or other confining means in which the color developing reactions can be conducted, on the patient's skin, whilst preventing spread of the reagents over too wide an area, and from which developed color can be presented to a means for determining and reporting a defined colored characteristic such as hue angle, e.g. a portable reflectance spectrometer, for examination and measurement. The container is suitably an adhesive strip provided with one or more cutout wells, initially provided with a protective backing to protect the adhesive. Preferably the container has at least two or three wells, so that control experiments can be conducted alongside test experiments. To facilitate the correct conducting of the test experiments and controlled experiments, the wells are conveniently made visually distinct from one another, e.g. of different shapes.

Figure 1 of the accompanying drawings illustrates such a container for use in the present invention, in the form of a test strip 10, of rectangular shape. The strip comprises a foam pad 10, with a layer 12 of skin compatible adhesive temporarily protected by a peelable release sheet 14. A first central well 16 for test purposes, of circular cross-section, extend through the foam body of the strip, and through the adhesive layer 12. A second well 18, for positive control purposes, of diamond cross-section, and a third well 20, for negative control purposes, of square cross-section, are similarly provided in the foam body of the strip 10, flanking the central well 16. The different visual shapes of th w lls assist the operator in conducting the tests, by aiding the correct choice of well for its

respective purpose.

The test is conducted preferably on the skin of the palm of the patient's hand. The container for the reagents, in the form of an adhesive strip, is temporarily adhered to the skin so that the open bottom of the wells contact the skin. Reagents are dropped into the wells, the color is developed in the wells, and then the color is read by the spectrophotometer without removing the strip from the skin. For this purpose, a specially designed spectrophotometer, constituting another feature of the invention, is used. The spectrophotometer transmits readings to a computer for analysis. The spectrophotometer is designed to ensure proper alignment over the test cell.

Accordingly, this aspect of the invention provides a spectrophotometer adapted to transmit signals from reading color reflectance of a test sample to a computer, the spectrophotometer having a body, a light emitting means in said body, an apertured lower portion of said body through which light emitting means may be directed to shine light, and a recess in the lower surface of said lower portion, adapted to fit over the welled test strip applied to a patient's skin surface to provide precise registry of the spectrophotometer and the test sample in a well of said welled test strip. Preferably, the lower portion of the spectrophotometer is hinged to the body, so that it can be conveniently fitted into proper registry with the test strip while in the open position, and then closed to the body of the spectrophotometer for taking measurements. Preferably also, the hinged lower portion and the body of the spectrophotometer are provided with electrical contacts to act as a switch, to turn on the light of the spectrophotometer when the lower portion is closed to the body of the spectrophotometer.

Figures 2, 3 and 4 of the accompanying drawings diagrammatically illustrate the spectrophotometer. It has a body 22, with electrical connection (not shown) to an appropriately programmed computer for analysis of the results read by the reader. A lower portion 24 is provided, hingedly connected at 26 to the body 22. The lower portion 24 is apertured at 28. A groove 30 extend across the

width of the under-surface of the lower portion 24, projecting upwardly from the lower most surface. The groove is closed at one side of the lower portion. The width of the groove 30 is designed to be a precise, tight fit over the test strip 10 shown in Figure 4. An end of test strip 10 is brought into registry with the closed end of the groove, when a reading is to be taken, and this, combined with the fit of the test strip 10 within the width of groove 30, provides precise registry of the test well 16 with the beam of light to be emitted from the body 22 of the reader, through the aperture 28. The spectrophotometer contains appropriate detector means for picking up reflectance signals from the sample in the well 16, and transmitting them for analysis and read out by the computer. The body 22 and the lower portion 24 are provided with respective electrical contacts 32, 34 which close as a switch when the lower portion 24 is closed to the body 22, thereby switching on the light for taking a measurement.

A specific test procedure will now be described, by way of specific, but non-limiting, example of the practice of the diagnostic test of the present invention.

The kit components include a dropper bottle containing detector solution (digitonin horseradish peroxidase cojugate in an aqueous buffered solution with less than 0.01% of bromonitrodioxane and methylisothiazolone as preservatives, 1.5mL), with a distinctive colored cap (green); a dropper bottle of more highly concentrated detector reagent solution, similarly buffered and preserved with less than 0.01% bromonitrodioxane and methylisothiazolone containing to act as a positive control (1.5ml), and with a distinctive cap (red); an indicator dropper bottle containing a solution of reagents (4.0 mL of 3,3',5,5'-tetramethylbenzidine, TMD, - hydrogen peroxide solution with 5% N,N-dimethylformamide as preservative) to react with the detector and PC reagents that are bound to skin cholesterol, to produce a blue-green color, being equipped with a distinctive cap (blue); foam pads as illustrated in Figur 1, to which the reagents may be added, alcohol swabs and appropriate directions for use. The chemical reagents are storage stable in a refrigerator at 2-8°C for extended

periods. The system also includes a spectrophotometerer as illustrated in Figures 2 and 3, connected to an appropriately programmed computer, and a calibration plaque for use with the spectrophotometer. The kit as supplied does not include a spectrophotometer, except perhaps when initially sold, the same spectrophotometer being re-used with subsequent "refill" kits.

Initially, the spectrophotometer is appropriately calibrated by inserting the calibration plaque into the jaws thereof, and closing it to illuminate the calibration plaque to feed calibration readings back to the spectrophotometer and computer. A signal is eventually received that calibration has been successfully completed.

The patient's hand is washed and rinsed thoroughly with soap and water and well dried. An outside portion of the palm of the patient's hand is then thoroughly cleaned with an alcohol swab, with sufficient scrub pressure to ensure thorough cleanliness. After allowing the hand to dry, release sheet 14 is removed from a test strip 10, which is then adhesively applied to the cleaned skin area of the patient's palm. The patient inverts the hand on a paper towel placed on a tabletop, and firmly presse down to ensure that the pad is properly adhered to the palm.

Next, the reagents are added to the respective reagent wells. One drop (42 μl) of detector solution is added to round test well 16, one drop of positive control solution is added to diamond-shaped cross-section well 18, but no liquid is added to the third, square well 20 at this point. Incubation of the added solutions is allowed to proceed for 1 minute, whilst the patient holds the test hand stationery. The patient then inverts the palm, and presses the foam pad on paper towel to remove liquid from the wells. Visual inspection is undertaken to ensure that the pad and the test wells are completely dry. Then the patient rests the hand on a flat surface with the palm facing upwards.

Next, one drop of indicator is added to all three wells, including the

square well previously unused, and reaction is allowed to proceed for 2 minutes, whilst the patient holds the hand stationery. Immediately afterwards, the reader 22 is put into position over test well 16, closed and a reading is taken of the color developed in the test well, transmitted to the spectrophotometer and analyzed by the computer, to give a read out of a value of hue angle.

Visual inspection of the positive control well 18 and the negative control well 20 is undertaken. If the liquid from the negative control well is colorless, and the liquid from the positive control well is colored, the test is valid. No quantitative measurements are taken of the color developed in the positive control. This is color developed from a very high solution concentration of reagent, to give a color with even very small amounts of cholesterol on the skin, and is simply indicative of the potency of the reagents, etc., for control purposes.

The residual liquid from the test strip is discarded, and the test strip is removed from the palm of the hand, followed by cleaning of the palm of the hand with an alcohol swab.

The spectrophotometer used in the present preferably measures absorbance of reflected light from the test sample, and converts it through a suitable algorithm to a value of hue angle. In the specific case of the development of color from horseradish peroxidase - TMD reaction described above, absorption at 450 nm, A_{450nm}, is a suitable measurement. Optical density of the absorbance at 450 nm has been experimentally determined to relate to hue angle through the relationship:

$$h°(\text{degrees}) = 490.45 \times A_{450\,nm} + 57.124$$

This relationship is determined by measuring optical density from a series of serially diluted reaction samples at 450 nm, measuring the hue angle of the same samples, and plotting the results on a regression curve to determine the relationship. Similar relationships can be worked out xperimentally in the same way for other chosen color developing tests using different enzyme - substrate

pairs which develop different colors, to allow optical density recordings to be transformed into hue angle determinations.

FIG. 1

Foam Pad

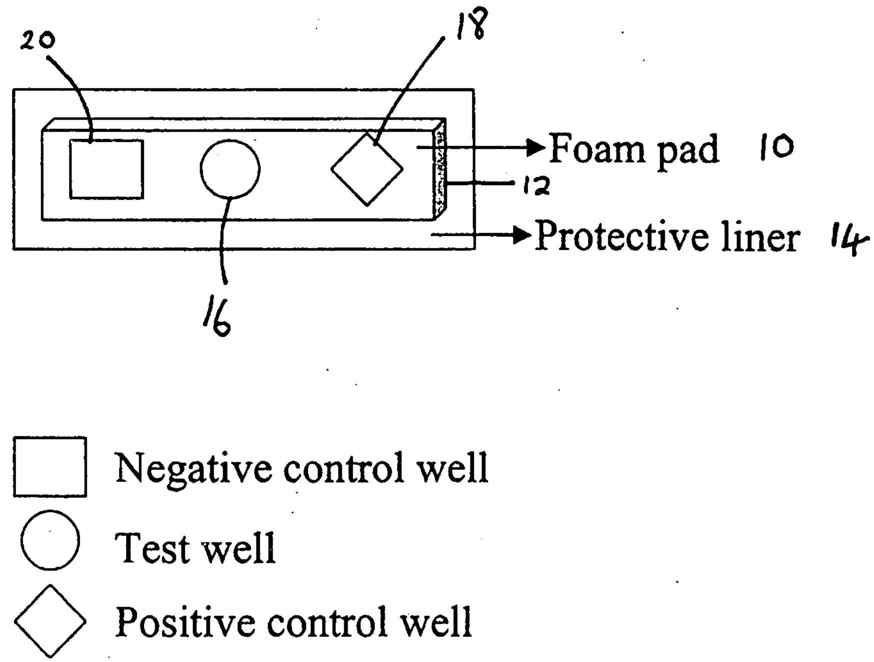

Fig. 2

Open Spectrophotometer on Palm

The spectrophotometer is placed on the foam pad on the palm at the end of the test such that the target window on the spectrophotometer aligns with the round center well on the foam pad.

Fig. 3

Closed Spectrophotometer on Palm

After proper positioning of the device, it is closed to activate the spectrophotometer. This results in measuring the patient's skin cholesterol (hue angle of the colour development in the test well).

Fig. 4.

"Shoe" of Spectrophotometer